

Action of Human Lysosomal Elastase on the Oxidized B Chain of Insulin

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(Received 5 July 1976)

The specificity of action of the lysosomal elastase of human neutrophil leucocytes on the oxidized B chain of insulin is similar to that of pig pancreatic elastase, but is more directed towards valine than alanine as the residue contributing the carboxyl group of the cleaved bond. The most susceptible bonds are Val-12–Glu-13 and Val-18–Cys(O₃H)-19. Other bonds hydrolysed are Ala-14–Leu-15, Ser-9–His-10 and Cys(O₃H)-7–Gly-8. Tables listing amino acid composition, N-terminal residue, and yields of isolated peptides have been deposited as Supplementary Publication SUP 50 075 (8 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1977) 161, 1.

The isolation and purification of two neutral proteinases from human spleen has been reported (Starkey & Barrett, 1976a). Detailed investigation of their properties has led to one of them being identified as a lysosomal elastase (EC 3.4.21.11) (Starkey & Barrett, 1976b), the other being a 'chymotrypsin-like' enzyme now called cathepsin G (Starkey & Barrett, 1976c).

The subject of the present investigation, and of the subsequent paper (Blow & Barrett, 1977), is the action of lysosomal elastase and cathepsin G on oxidized insulin B chain, in order to define the specificity of these two different enzymes on a polypeptide substrate.

The properties of pig pancreatic elastase are well established (Hartley & Shotton, 1971), and its specificity of action on the insulin B chain has been described by Sampath Narayanan & Anwar (1969), but this is the first report on the specificity of an elastase present in lysosomes of neutrophil leucocytes and possibly some other cells. The lysosomal elastase is capable of degrading proteoglycan, collagen and several other proteins, as well as elastin (Starkey & Barrett, 1976b).

Experimental

Materials

Human leucocyte elastase prepared by the method of Starkey & Barrett (1976a) was given by Dr. P. M. Starkey.

Oxidized B chain of bovine insulin was from Schwarz/Mann, Orangeburg, NY, U.S.A., and was

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used without further purification. Bio-Rad AG 50W-X2 (200–400 mesh) cation-exchange resin was from Bio-Rad Laboratories, St. Albans, Herts., U.K.

Methods

Separation of digestion products of insulin B chain. Oxidized B chain (10 mg in 1.0 ml of 0.1 M-NH₄HCO₃, pH 8.5) was digested with 25 µg of elastase for 30 min or 4 h at 37°C. The reaction was stopped by lowering the pH to 3.0 with 6 M-HCl. Initial separation of peptides was on a cation-exchange resin by the method of Schroeder (1972). The complete digest was applied to a column (50 cm × 1 cm) of Bio-Rad AG 50W-X2 (200–400 mesh), equilibrated with pyridine/acetic acid buffer, pH 3.1 (64.5 ml of pyridine and 1114 ml of acetic acid diluted to 4 litres with water; 0.2 M in pyridine). The column was developed with a gradient made by mixing 83 ml of the 0.2 M-buffer, pH 3.1, with 166 ml of 2.0 M-buffer, pH 5.0 (645 ml of pyridine and 573 ml of acetic acid diluted to 4 litres with water; 2.0 M in pyridine), in a two-chamber gradient mixer with a ratio of diameters of 1:1.4 (Schroeder, 1972). The column was pumped at a flow rate of 10 ml/h at 38°C, and 1 ml fractions were collected. The peptides were located in the effluent by use of ninhydrin after alkaline hydrolysis (Hirs, 1967). Tubes containing separated peptides were combined and dried down by rotary evaporation under reduced pressure at 35°C. The separated fractions were then re-dissolved in 150 µl of 0.1 M-acetic acid and kept frozen. Further purification was by high-voltage electrophoresis. The separated bands were located by staining guide-strips with a cadmium/ninhydrin reagent (Barrolier *et al.*, 1957) and eluted with water.

Amino acid analysis. The peptides were hydrolysed with 6 M-HCl, containing 1% (w/v) phenol, in

evacuated sealed tubes for 18 h at 105°C and run on a Locarte amino acid analyser, by use of a one-column system with sodium buffers, as suggested in the manufacturers' instructions.

Calculation of yields. All yields reported were calculated from the analyses of the purified peptides.

Identification of N-terminal residues. N-Terminal residues were identified by the dansyl chloride method, with separation of the dansylamino acids on polyamide sheets (Gray, 1972).

High-voltage electrophoresis. Peptides were purified analytically on Whatman no. 1 paper, and preparatively on Whatman 3MM paper, in formic acid/acetic acid/water (25:78:897, by vol.), pH 1.9, at 100 V/cm for 20 min or 50 V/cm for 40 min respectively, on a Camag (4132 Muttenez, Switzerland) flat-bed apparatus unless otherwise specified.

Results

Purity of insulin B-chain preparation

Duplicate samples of 75 µg (23 nmol, assuming the material is 100% pure) of B chain were hydrolysed for 18, 48 and 72 h, with 6M-HCl containing 1% (w/v) phenol in evacuated sealed tubes at 105°C. Amino acids corresponding to 16–17 nmol of polypeptide were recovered, regardless of the duration of the hydrolysis period. None of the amino acids showed a significantly decreased recovery after longer hydrolysis, and only two were recovered at less than 95% of the theoretical yield, if it was assumed that 16.5 nmol of polypeptide had been hydrolysed (cysteic acid, 81%; tyrosine, 89%). It was concluded that B chain preparation was 73–75% peptide, and calculations of yields of peptides are based on this assumption.

Digestion for 4 h

Preliminary experiments in which products were separated by high-voltage electrophoresis indicated that no undigested B chain remained after 4 h of digestion under the specified conditions.

Five major components were isolated from the digest by ion-exchange chromatography; high-voltage electrophoresis indicated that four of these were pure, and hence they were hydrolysed for amino acid analysis and their N-terminal residues identified (data in Supplementary Publication SUP 50075). From these data the peptides were identified as containing residues 19–30 (yield 22.4%), 1–12 (yield 18.5%), 13–18 (yield 14.7%) and 1–14 (yield 9.5%). The fifth peptide was further purified by preparative high-voltage electrophoresis, and after analysis for amino acid composition and N-terminal residue was identified as containing residues 15–18 (yield 8%).

These peptides account for the whole of the B-chain structure, and indicate major cleavages between valine-18 and cysteic acid-19, and between valine-12

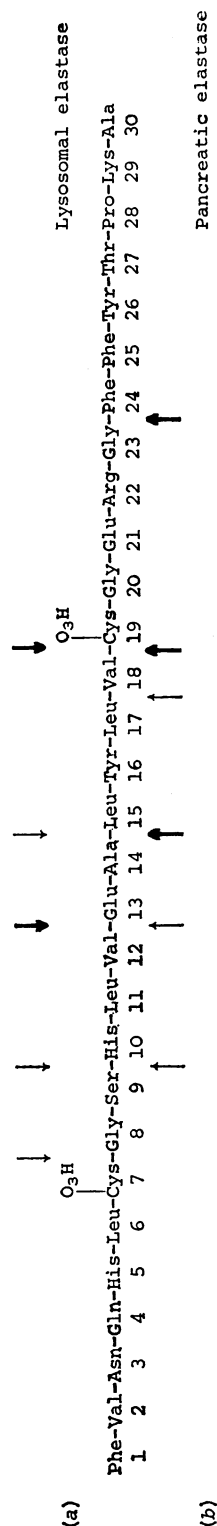


Fig. 1. Specificity of action of human lysosomal (a) and pig pancreatic elastase (b) on insulin B chain. Large arrows, major sites; small arrows, minor sites. Data for pig pancreatic elastase are from Sampath Narayanan & Anwar (1969).

and glutamic acid-13. As judged by the data on yields, the cleavage between alanine-14 and leucine-15 is a relatively minor one (see Fig. 1).

Digestion for 30 min

In order to check the relative rate of cleavage at each site, a digestion was done for 30 min and products were fractionated as described above. From the position of peaks on the ion-exchange chromatogram and by comparison of mobilities of the products after high-voltage electrophoresis the four major peptides could again be identified, and this was confirmed by amino acid analysis. Peptides containing residues 19–30 (yield 34%), 1–12 (yield 19.5%), 13–18 (yield 5.4%) and 1–14 (yield 4.4%) were found. The yield of peptide 1–12 is high and essentially identical for the two time-periods, indicating that this peptide is produced by a major cleavage site. The peptide containing residues 18–30 was found in higher yield after 30 min digestion than after 4 h, again indicating that it is produced by a major cleavage, and suggesting that it may be subject to further attack, though peptides resulting from such attack were not identified after 4 h digestion. By contrast, peptides containing residues 1–14 and 15–18 were found in higher yield after 4 h than after 30 min, indicating a relatively minor cleavage site between alanine-14 and leucine-15.

Comparison of the isolated peptides with a complete digest (4 h) on high-voltage electrophoresis suggested that some further peptides were being produced in low yield. One of these was isolated after high-voltage electrophoresis, and had a composition suggesting it represented residues 10–12 and had been produced by a cleavage between serine-9 and histidine-10, in addition to the major cleavage that produced the peptide containing residues 1–12.

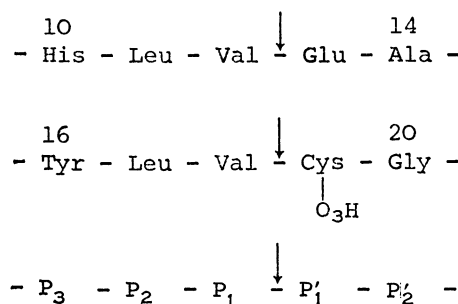
In order to check the susceptibility of the bond between residues 9 and 10 and to test the possibility that the peptide containing residues 19–30 may undergo further attack, as it does with pancreatic elastase, peptides containing residues 1–12 and 19–30 were digested for 4 and 24 h, followed by high-voltage electrophoresis. After 4 h, there was evidence of some new components in each digest, but after 24 h it was clear that substantial digestion of peptide containing residues 1–12 had occurred, whereas peptide containing residues 18–30 had not undergone attack. In particular there was no evidence of a major cleavage site between glycine-23 and phenylalanine-24 as was found for pancreatic elastase (see Fig. 1).

The products of digestion of peptide containing residues 1–12 after 24 h were separated by high-voltage electrophoresis, and characterized by amino acid analysis. Peptides containing residues 1–9 (38% yield), 10–12 (22% yield), 1–12 (16% yield) and 8–12 (13% yield) were found. This gives good evidence of a

further minor cleavage site between serine-9 and histidine-10, as was found for pancreatic elastase, and some evidence for cleavage between cysteic acid-7 and glycine-8 (not found for pancreatic elastase).

Discussion

The major points of cleavage of the insulin B chain by lysosomal elastase are between valine-12 and glutamic acid-13, and between valine-18 and cysteic acid-19. Inclusion of adjacent amino acid residues shows that these two sites are very similar.



The presence of leucine in the P_2 subsite may be significant, since it is also present in this position in the otherwise anomalous minor site between cysteic acid-7 and glycine-8.

Both the above major sites are also cleaved by pancreatic elastase, though the site between valine-13 and glutamic acid-14 is considered to be a relatively minor one (Sampath Narayanan & Anwar, 1969). Of the two major sites for the pancreatic enzyme, one, between glycine-23 and phenylalanine-24, is not cleaved at all by the lysosomal enzyme, and the other, between alanine-14 and leucine-15, is a relatively minor site for this enzyme. The conclusion can thus be drawn that valine is preferred to alanine in the P_1 subsite for the lysosomal enzyme as against the pancreatic enzyme. Work using active site-directed inhibitors, such as chloromethyl ketone derivatives of low-molecular-weight peptides (J. C. Powers & R. J. Whitley, personal communication) and with substrate analogues (J. C. Powers & F. Gupton, personal communication), supports this assertion. It had also been predicted that the P_2 subsite should contain a proline or leucine residue, for maximum reactivity of such inhibitors (Tuhy & Powers, 1975), which is confirmed for leucine by the present work with the insulin B chain as substrate.

I thank Professor J. Travis and other members of the Tissue Physiology Department for helpful discussions. I thank the Nuffield Foundation for financial support.

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